Lipids of Virus Infected Cells. II. Lipid Analysis of HeLa Cells Infected with Vaccinia Virus.* (28257)

CHARLES R. GAUSH† AND J. S. YOUNGNER
Department of Microbiology, School of Medicine, University of Pittsburgh, Pittsburgh, Pa.

In a previous study(1), we found that infection of chorioallantoic membranes (CAM) with vaccinia virus produced significant changes in the lipid composition of infected cells. Decreases in concentration of choline and ethanolamine phosphatides were reported which were accompanied by marked increases in content of monophosphoinositol and triglyceride. From the evidence presented, it appeared that the metabolic pathways for synthesis of phospholipids(2) had been affected. On the other hand, it was considered possible that the changes were caused by the accumulation of certain extracellular serum lipids(3,4) in the cells injured by virus.

The present report describes an attempt to resolve this problem by analyzing the lipids of cells infected with virus in the absence of extracellular lipids.

Materials and methods. HeLa cells were grown in 32 oz bottles containing Eagle's Medium with 8% bovine serum added. Cultures incubated at 37° for 7 days developed a confluent monolayer which contained 2-3 $\times$ 10^7 cells. The medium was removed from the cultures and replaced with Eagle's maintenance medium without serum and inoculated with vaccinia virus at a multiplicity of 1.0. At intervals up to 48 hours after inoculation, groups of infected and control cultures (80-100 bottles per group) were harvested and the cells separated by centrifugation. After homogenization in phosphate buffered saline (PBS), aliquots were removed for infectivity and hemagglutinin assays (Fig. 1). The remaining homogenate was used for the lipid analyses.

Infectivity and hemagglutinin assays of virus suspensions were performed as described previously(1).

Total lipids were extracted from virus infected and control cell homogenates with absolute ethyl alcohol and diethyl ether. After standing at room temperature for 2 hours, the residue was sedimented by centrifugation and extracted twice more with Bloor's solvent. The lipid extract was clarified by filtration through glass fiber paper (Reeve-Angel #934-AH), reduced to a small volume and reextracted with ether. The second extract was washed with demineralized water (20% v/v), the ether removed by evaporation under nitrogen and the lipids dried *in vacuo* over Drierite for 18 hours at room temperature.

Neutral lipids (NL) were separated from phospholipids (PL) on columns of silicic acid (25 mg total lipid/g "Unisil" silicic acid). The neutral lipids were eluted with 300-400 ml chloroform and the phospholipids with 300-400 ml methanol.

Neutral lipids were separated into 5 classes using the method of Horning *et al.*(5). The lipids in each fraction were identified by chromatography on silicic acid coated glass fiber paper with diisobutyl ketone:n-heptane (9:1), a modification of the method of Marinetti(6). Lipid spots were located on the dried chromatograms by staining with protoporphyrin (7) and viewing under ultraviolet light (3660 Å).

Phospholipids were eluted from silicic acid (Bio-Rad) columns as described by Hanahan *et al.*(8) (Fig. 2). Each fraction was hydrolyzed in 6N HCl at 120°C for 18 hours and the aqueous phase chromatographed on Whatman #1 paper with n-propanol, ethyl acetate, and water in the ratio 7:1:2(9). Serine and ethanolamine were identified by ninhydrin spray, glycerol and inositol by alkaline silver nitrate spray(10). Choline was identified by the method of Levine and Chargaff(11). Infra-red spectra were obtained with a Beckman IR-7 instrument using thin films of un-

* From a doctoral thesis submitted by the senior author to School of Medicine, Univ. of Pittsburgh.

This study was aided by grants from U. S. Public Health Service.

† Present address: Dept. Microbiology, School of Medicine, State Univ. of South Dakota, Vermillion.

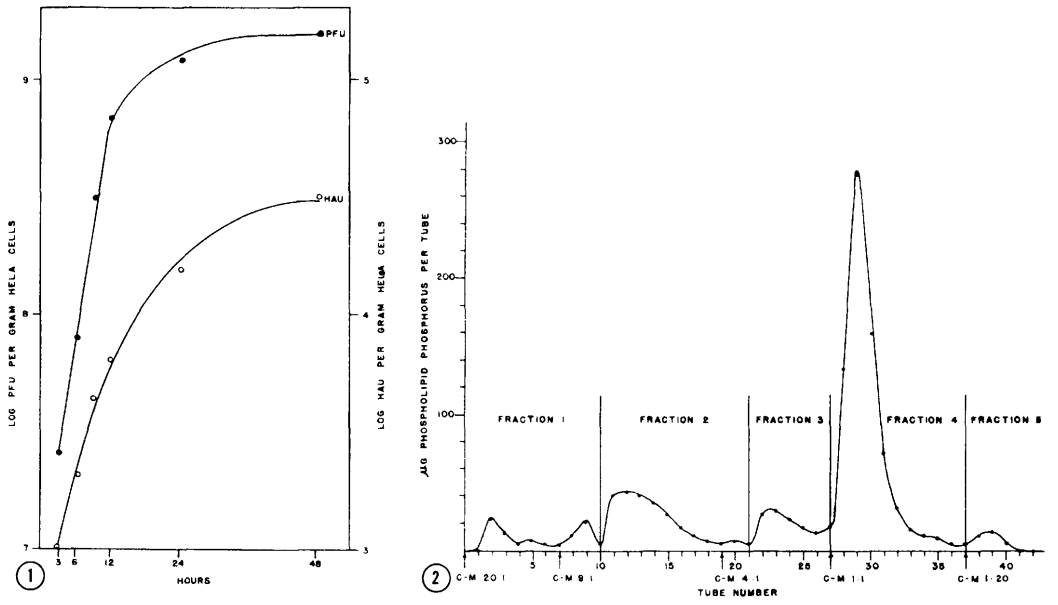

FIG. 1. Replication of vaccinia virus in HeLa cell monolayers. Cultures were inoculated with 6×10^7 pfu virus (multiplicity of 2.0) in Eagle's medium without serum.

FIG. 2. Separation of phospholipids from vaccinia virus infected HeLa cells on a silicic acid column.

hydrolyzed lipid on salt plates or in KBr pellets.

Incorporation of P^{32} into cellular lipids was studied under identical conditions with cultures that had been pre-incubated with 124.2 μC P^{32} 4 hours prior to infection with 6×10^7 plaque forming units of virus. The isotope was not removed before virus inoculation. These cultures were harvested at 6 and 24 hours which corresponded to the beginning and end of the logarithmic phase of virus replication (Fig. 1).

Results. These analytical procedures revealed that fraction 1 (Fig. 2) contained phosphatidyl serine (PS) but that some phosphatidyl ethanolamine (PE) was also present. The second peak in fraction 1 was also PS and was an artifact frequently seen in stepwise elutions schemes. Fraction 2 was identified as PE and fraction 3 as phosphatidyl inositol (PI). Infra-red analysis indicated that fraction 4 was composed of phosphatidyl choline (PC) but that a small amount of sphingomyelin was also present. The IR spectrum of the phosphatide from fraction 5 was the same as that of a synthetic PC but absorption bands at 1650 and 3400 cm^{-1} indicated the presence of trace amounts of another compound, probably diphosphoinositide. Fractions 4 and 5 were combined and reported as choline phosphatides (CP) in Tables I and II.

The composition of the total lipid fraction of HeLa cells at intervals after infection with vaccinia virus is presented in Table I. The differences between the average value after 6 hours and initial value (0 HR) in normal and infected cultures indicated that the greatest changes occurred in the sterol ester, PS and PE fractions. The concentration of these compounds, with the exception of PS, decreased between 0 and 6 hours in both normal and infected cultures and did not change further upon incubation for an additional 42 hours. The concentration of PS increased between 0 and 6 hours and remained elevated for 42 hours which suggests an inverse proportionality between PS and PE, a fact which is substantiated by the overlapping of these fractions. Other apparent changes could be attributed to overlapping fractions and incomplete recovery of lipids. It was concluded that the lipid composition of HeLa cells was not altered by infection with vaccinia virus under these conditions.

Separation of labeled phosphatides into in-

TABLE I. Composition of the Lipid Fraction of HeLa Cells at Intervals During Virus Replication Expressed as % of Total Lipid Extracted.

Time, hr	Hyd	SE	Tg	S	M & Dg	PS	PE	PI	CP	% Rec.
					Normal cells:					
0	.87	3.40	3.77	11.08	3.53	8.03	18.39	6.63	45.16	100.86
6	1.72	1.20	1.72	10.49	4.28	8.92	12.72	9.10	47.11	97.26
12	1.20	.92	2.62	11.61	4.18	8.58	14.82	8.77	47.36	100.06
24	1.15	1.23	2.70	10.64	3.93	11.06	16.06	5.47	43.38	95.62
48	.97	1.07	2.81	13.94	3.80	10.61	8.99	2.34	49.65	94.18
Avg*	1.26	1.11	2.46	11.67	4.05	9.79	13.15	6.42	46.88	96.78
0 hr minus avg	−.39	2.29	1.31	−.59	−.52	−1.76	5.24	.21	−1.72	
					Infected cells:					
6	1.73	1.91	2.18	11.26	4.26	9.28	14.94	7.91	44.74	98.21
12	1.40	.88	2.54	8.34	3.86	9.52	15.39	9.08	49.12	100.13
24	1.69	1.69	3.84	10.18	5.09	10.69	14.37	5.36	42.88	95.79
48	1.49	1.58	3.41	12.87	4.23	10.91	15.98	5.83	42.83	99.13
Avg	1.58	1.52	2.99	10.66	4.36	10.10	15.17	7.05	44.89	98.32
0 hr minus avg	−.71	1.88	.78	.42	−.83	−2.07	3.22	−.42	.27	

Hyd, hydrocarbons; SE, sterol esters; Tg, triglycerides; S, sterols; M & Dg, mono- & diglycerides; PS, phosphatidyl serine; PE, phosphatidyl ethanolamine; PI, phosphatidyl inositol; CP, choline phosphatides.

* Does not include 0 hr values.

dividual phospholipids (Table II) showed that the specific activity of each fraction was elevated at 6 hours but 24 hours after infection, the specific activity of PI was 73% higher than in control cells and was the only phosphatide with significantly elevated activity.

Discussion. Cornatzer *et al.*(12) reported a progressive increase in the synthesis of phospholipids except PS during poliovirus infection of HeLa cells. In our experiments, the P^{32} specific activities of the separated phospholipids from infected cells were similar to those observed by Cornatzer(12), but we found that specific activities of the control cell phospholipids also increased during the 24 hour interval. This finding was unexpected since the P^{32} was added to the cultures 4 hours prior to virus inoculation which allowed sufficient time for isotope equilibration (13). However, the observed increase in specific activity of control cell lipids may be a reflection of the change to maintenance medium without serum.

Significant changes in the lipid composition of virus infected cells *in vitro* were not observed, which is in contrast to the findings of the *in ovo* system described previously(1). Failure of the infected cells to accumulate lipid appears to be related to the absence of serum lipids in the tissue culture medium. The data also indicate that the vaccinia-HeLa cell system was similar to other *in vitro* virus-cell systems with respect to phospholipid me-

TABLE II. Composition of Vaccinia Virus Infected HeLa Cell Phospholipids and Specific Activities of Individual Phosphatides.

	Phosphatidyl serine		Phosphatidyl ethanolamine		Phosphatidyl inositol		Choline phosphatides		% PL recovered
	% of TL	S.A.*	% of TL	S.A.	% of TL	S.A.	% of TL	S.A.	
6 hr normal	12.78	44.90	17.07	29.90	5.48	44.10	41.76	10.60	103.23
6 hr infected	14.77	64.60	9.00	40.40	3.24	68.70	36.84	21.50	85.42
% change		43.87		35.11		55.78		102.83	
24 hr normal	17.33	131.90	12.95	127.95	3.96	134.20	40.97	106.30	91.81
24 hr infected	12.70	157.20	14.47	124.00	5.16	232.10	42.63	122.30	95.84
% change		19.18		−3.08		72.95		15.05	

* Specific activity $= \frac{\text{Counts/min}/\mu\text{g phosphorus}}{100}$.

tabolism. An early increase in phospholipid synthesis as indicated by P^{32} incorporation may be a general phenomenon occurring in all virus infected cells but sufficient data are not available to bear this out.

The significance of the elevated specific activity of phosphatidyl inositol 24 hours after infection is not clear but may be associated with the egress of mature virus from the cell or with an increase in transport of nutrients into the severely damaged cells.

Summary. The lipid composition of HeLa cells infected with vaccinia virus as well as non-infected cells is described. The results indicate that virus infection in a serum-free medium does not alter the lipid composition of the cells. However, the incorporation of P^{32} into the phospholipids of virus infected HeLa cells was greater than that observed in non-infected cells, a finding that appears to be generally applicable to virus infected cells.

1. Gaush, C. R., Youngner, J. S., *Virology,* in press.

2. Rossiter, R. J., Strickland, K. P., *Lipid Metabolism,* Ed., K. Bloch, John Wiley & Sons, New York, 1960.

3. Bailey, J. M., Gey, G. O., Gey, M. K., PROC. SOC. EXP. BIOL. AND MED., 1959, v100, 686.

4. Bensch, K. G., King, D. W., Socolow, E. L., *J. Biophys. Biochem. Cytol.,* 1961, v9, 135.

5. Horning, M. G., Williams, E. A, Horning, E. C., *J. Lipid Research,* 1960, v1, 482.

6. Marinetti, G. V., Stotz, E., *Biochim. et Biophys. Acta,* 1960, v37, 571.

7. Sulya, L. L., Smith, R. R., *Biochem. and Biophys. Res. Comm.,* 1960, v2, 59.

8. Hanahan, D. J., Dittmer, J. C., Warashina, E., *J. Biol Chem.,* 1957, v228, 685.

9. Cerbulis, J., *Anal. Chem.,* 1955, v27, 1400.

10. Trevelyan, W. E., Procter, D. P., Harrison, J. S., *Nature,* 1950, v166, 444.

11. Levine, C., Chargaff, E., *J. Biol. Chem.,* 1951, v192, 465.

12. Cornatzer, W. E., Sandstrom, W., Fischer, R. G., *Biochim. et Biophys. Acta,* 1961, v49, 414.

13. Miroff, G., Cornatzer, W. E., Fischer, R. G., *J. Biol. Chem.,* 1957, v228, 255.

Received February 25, 1963. P.S.E.B.M., 19663, v112.

Age-Related Changes in the Behavior of Diethyleneglycol Succinate Polyester Columns. (28258)

K. IMAICHI, G. MICHAELS AND G. FUKAYAMA (Introduced by L. Kinsell)
Institute for Metabolic Research, Highland-Alameda County Hospital, Oakland, Calif.

During the course of gas-liquid chromatographic studies with fish oil fractions(1) containing considerable amounts of long chain highly unsaturated fatty acids, it became apparent that as the succinate polyester column aged, progressive changes occurred in the fatty acid patterns.

Magidman(2), using butter fat, reported that as the succinate polyester column was used, the stationary phase appeared to become less polar in character and the retention time for the unsaturated esters changed at different rates than for the saturated esters.

A more precise definition of the changes which occur in relation to aging seemed desirable. Therefore the following studies were carried out.

The polyester diethyleneglycol succinate was prepared according to the procedure of Koroly and Beavers(3) as modified by Craig and Murty(4). The cross-linking agent, diglycerol, was omitted since it was not necessary for the synthesis of this polyester. We also eliminated the catalyst, zinc chloride, because trace amounts left after treatment with Amberlite IR-120 resulted in shorter column life. Elimination of the catalyst made it necessary to increase the initial heating time at 160°C from 4 to 6 hours. The columns were prepared as follows: the polyester was dissolved in acetone and the solid support Celite 60-100 mesh was added in a ratio of 1:4. This was then dried in a rotary evaporator under reduced pressure. The dried material was then added to the column. Good packing